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Syntheses and Preliminary Biological Studies of Four *meso*-Tetra[(*nido*-carboranylmethyl)phenyl]porphyrins

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Abstract—Two *meso*-tetra[(*nido*-carboranylmethyl)phenyl]porphyrins (*para*- and *meta*-regioisomers) and their corresponding Zn(II) complexes have been synthesized with the aim of studying the effect of carborane distribution and metalation on the biological properties of this series of compounds. In vitro cell toxicity, uptake/efflux, and subcellular localization using rat 9L, mouse B16 and/or human U-373MG cells were evaluated. All four amphiphilic porphyrins display very low cytotoxicities and time- and concentration-dependent uptake by cells, which is influenced by serum proteins. Preliminary subcellular localization studies suggest that one of these compounds localizes in close proximity to the cell nucleus. All four *nido*-carboranylporphyrins show promise as boron-carriers for the boron neutron capture therapy of cancers, particularly the metal-free *nido*-carboranylporphyrins **5** and **12**, which are able to deliver higher amount of boron to cells in vitro than the corresponding zinc complexes.

Introduction

Boron neutron capture therapy (BNCT)[1,2] is a binary modality for cancer treatment, based on the capacity of ^{10}B nuclides to capture low-energy (thermal) neutrons, which produces high linear energy transfer (LET) particles, $^{4}He^{2+}$ (α-particle) and $^{7}Li^{3+}$, bearing approximately 2.4 MeV of kinetic energy [eq. (1)]:

$$^{10}B + 1_n \rightarrow {}^{7}Li^{3+} + {}^{4}He^{2+} + \gamma + 2.4MeV \quad (1)$$

The α-particles and ^{7}Li nuclei produced in the nuclear reaction are extremely cytotoxic and display only a limited distance of travel in tissue (approximately one cell diameter, 9 and 5 μm, respectively). Therefore in principle, selective and efficient destruction of ^{10}B-containing malignant cells in the presence of normal, ^{10}B-free cells, can be achieved in BNCT. The advantages of such a localized therapeutic approach to cancer treatment are obvious since undesirable side effects that are common in other types of treatments, such as chemotherapy and radiotherapy can be minimized. In addition, the recurrence of metastatic tumors can potentially be controlled and/or eliminated using such a selective therapeutic modality. BNCT is particularly attractive for the treatment of malignant brain tumors,[3] which are usually highly infiltrative of normal brain and where selective destruction of tumor cells could dramatically increase patient life quality and expectancy.

The ultimate success of BNCT depends upon whether thermal neutrons and adequate amounts of ^{10}B nuclei can be selectively and effectively delivered to tumor cells. Since the production of low-energy neutrons with high beam quality has been achieved by modern nuclear reactors, the main unsolved problem in BNCT centers on the development of new ^{10}B-carriers, capable of selectively delivering substantial amounts of ^{10}B atoms to tumors.[4] Although the natural abundance of ^{10}B is 20%, this isotope can be synthetically incorporated in enriched compounds at the 95–96% level. A promising new BNCT agent should: (1) selectively target tumor cells, (2) deliver therapeutic and predictable concentrations of ^{10}B atoms to tumors, (3) display low toxicity, (4) preferentially localize within malignant cells, and in particular in or near the nucleus, (5) be rapidly cleared from blood and normal tissues, while displaying relatively long retention times in tumors, (6) be readily synthesized in pure form, and (7) be easily monitored in tissue using direct methods, for evaluation of ^{10}B-localization sites and quantification, in order to accurately determine the radiation microdosimetry. It has been estimated that the

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concentration of ^{10}B necessary for effective BNCT is 15–30 μg of ^{10}B per gram of tumor, depending upon the precise location of the ^{10}B atoms, and that high tumor: blood and tumor:normal tissue boron concentration ratios would significantly increase BNCT effectiveness.[5] In recent years several research groups[6,7] have developed a variety of new ^{10}B-carriers with improved tumor selectivity and retention times over the two BNCT capture agents currently undergoing clinical trials, disodium mercapto-*closo*-dodecaborate (BSH)[8] and L-4-dihydroxyborylphenylalanine (BPA).[9] Of all the new boron-delivery agents, porphyrins appear to be particularly promising tumor-selective compounds because of their demonstrated tendency to accumulate in neoplastic tissue.[10,11] This property of porphyrins provides the basis for their use in another binary therapeutic method, photodynamic therapy (PDT) of tumors,[12,13] which relies on the selective uptake of a photosensitizer in tumor tissues, followed by generation of singlet oxygen and other cytotoxic species upon irradiation with light. Porphyrins and their diamagnetic metal complexes are highly fluorescent; this also provides a significant advantage over other boron carriers since it enables the detection of tumor cells using confocal fluorescence microscopy[14,15] and the determination of ^{10}B localization in tumors and surrounding tissues. Intracellular boron distribution and quantification play an important role in determining the dose and length of neutron radiation, as well as the success of BNCT treatment.[16]

Several boron-containing porphyrin derivatives have been reported in the last decade for application in BNCT.[17–30] The results achieved with some of these compounds indicate that boronated porphyrins retain their ability to selectively accumulate within tumor cells, and are able to deliver high concentrations of boron to tumors (>30 μg ^{10}B/g tumor). High tumor to blood ratios (>5:1) and tumor to normal tissue ratios (>10:1) have been achieved in small animals with some carboranyl-containing porphyrins. Furthermore, the favorable intracellular localization and long retention times of some boronated porphyrins in tumor cells makes them highly attractive for application in BNCT. However, several questions concerning possible in vivo hydrolytic stability of the carborane–porphyrin linkages, their sometimes excessively hydrophobic character, and the influence of the nature and distribution of the carborane residues about the porphyrin macrocycle have arisen, and remain the topic of continuous research. We have prepared new series of carborane-containing porphyrins via expeditious synthetic methodologies, bearing novel and chemically stable carbon–carbon linkages between the porphyrin macrocycles and the carborane residues.[31] Herein, we describe our preliminary results from the in vitro evaluation of one series of these compounds.

Results

Porphyrin syntheses

The amphiphilic *nido*-carboranylporphyrins used in this study were synthesized from readily available starting materials, as indicated in Schemes 1 and 2.[31,32] The *ortho*-carboranylporphyrins **4** and **11** were synthesized from the corresponding 4- and 3-bromomethylbromobenzene (**1** and **8**) in, respectively, 16 and 18% overall yields. The metalation of porphyrins **4** and **11** was achieved using $ZnCl_2$ in dichloromethane and tetrahydrofuran (THF) in a 10:1 ratio, and in the presence of pyridine. The recently reported[33] molecular structure of the zinc porphyrin **6** is shown in Figure 1. This series of structurally related carboranylporphyrins was obtained with the aim of studying the effect of metalation and of carborane distribution about the porphyrin macrocycle, on the biological properties of these compounds.

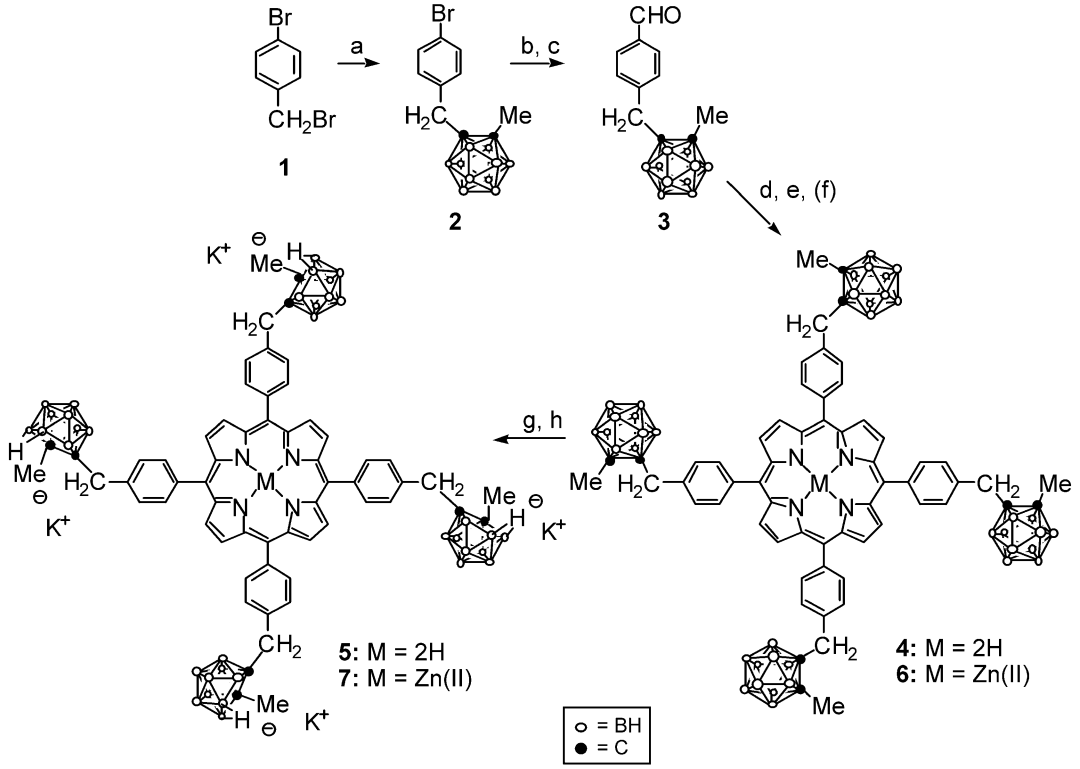
Stock solutions of carboranylporphyrins **5**, **7**, **12**, and **14** were prepared by weighing an exact amount of porphyrin in crystalline form and dissolving in 100% DMSO; subsequent dilutions were done directly into the culture medium, just prior to administration to cells. The porphyrin stock solutions were stored in 100% DMSO at 5 °C and protected from exposure to light. The concentration of the porphyrin solutions obtained from dilution of the stocks was verified both spectrophotometrically and by inductively-coupled argon plasma mass spectrometry (ICP-MS).

Amphiphilic character

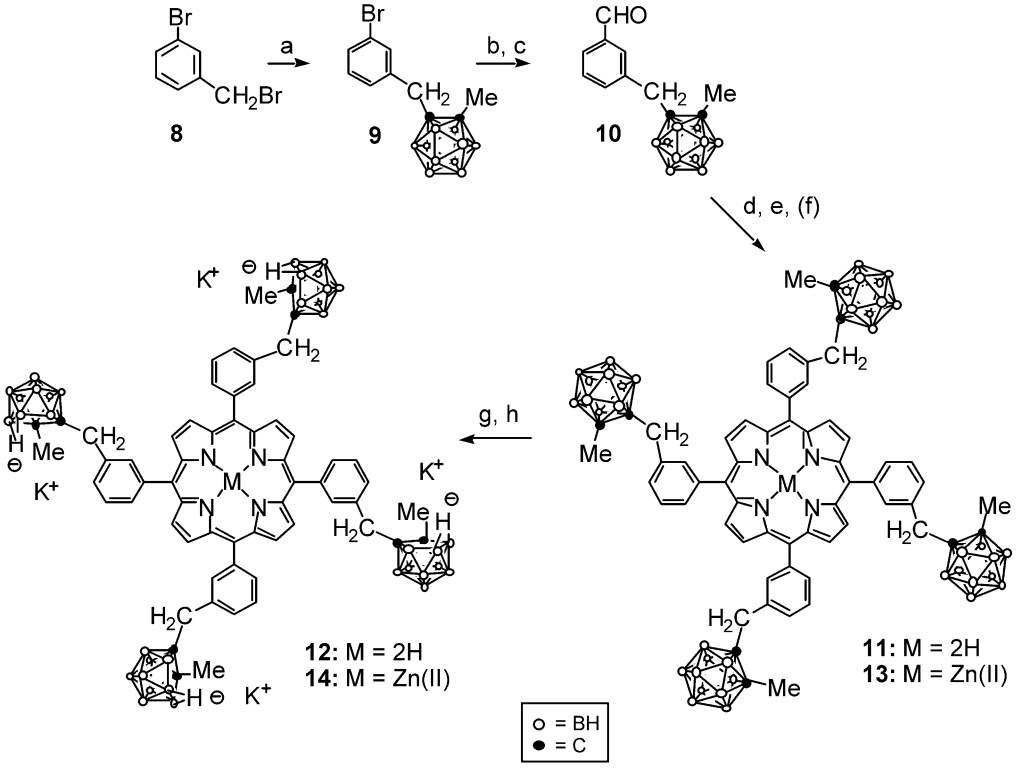
The degrees of amphiphilicity of the water-soluble *nido*-carboranylporphyrins were estimated from their distribution between 1-octanol and a phosphate buffered solution at pH 7.4.[34] For comparison purposes, the partition coefficients (P) for protoporphyrin IX (PP-IX) and *meso*-tetra(4-carboxyphenyl)porphyrin [$TPP(CO_2H)_4$] were also determined. The results obtained revealed that porphyrin **5** displays the highest hydrophobic character of all the *nido*-carboranylporphyrins studied: **5**, $P=65$; **12**, $P=50$; **7**, $P=44$; **14**, $P=41$; PP-IX, $P=14$; $TPP(CO_2H)_4$, $P=0.1$.

Cytotoxicity

After a 2-h time exposure to each of the *nido*-carboranylporphyrins **5**, **7**, **12**, and **14** at concentrations up to 250 μM, none of the three cell types studied showed any inhibition of proliferation when monitored for 72 h post-treatment, in the dark. The cell morphology and the proportion of mitotic cells as determined by phase microscopy, was indistinguishable from untreated control cells. In contrast, a 24-h time exposure did inhibit proliferation of both 9L and U-373MG cells at the highest concentrations tested. Estimated IC_{50}s (treatment that results in a 50% inhibition of cell proliferation compared to controls) from dose–response curves were found to be nearly identical for both cell types, and are given in Table 1. Whereas the metal-free porphyrins **5** and **12** display identical IC_{50} values, their Zn(II) complexes were found to be approximately 20% less toxic to the above cell lines. Mouse B16 cell viability was not affected, even at concentrations of 250 μM of all *nido*-carboranylporphyrins for 24 h. The time-dependent toxicity was more dramatic in the phototoxicity experiments. For carboranylporphyrin **5**, irradiation with red light



Scheme 1. (a) 1-lithium-2-methyl-*o*-carborane, DME, reflux 10 h (75%); (b) *n*-BuLi, THF, −78 °C, then DMF; (c) 5% HCl (62% from **2**); (d) pyrrole, TFA, in CH_2Cl_2; (e) *p*-chloranil (32% from **3**); (f) $ZnCl_2$ in CH_2Cl_2/THF/pyridine (86–90%); (g) pyridine/piperidine (3:1), 36 h; (h) resin Dowex 50WX2-100 in K+ form (94–96% from **4** or **6**).



Scheme 2. (a) 1-lithium-2-methyl-*o*-carborane, LiI, THF, rt, 12 h (69%); (b) *n*-BuLi, THF, −78 °C, then DMF; (c) 2 N HCl (79% from **9**); (d) pyrrole, TFA, in CH_2Cl_2; (e) *p*-chloranil (33% from **10**); f) $ZnCl_2$ in CH_2Cl_2/THF/pyridine (89–91%); (g) pyridine/piperidine (3:1), 36 h; (h) resin Dowex 50WX2-100 in K+ form (94–96% from **11** or **13**).

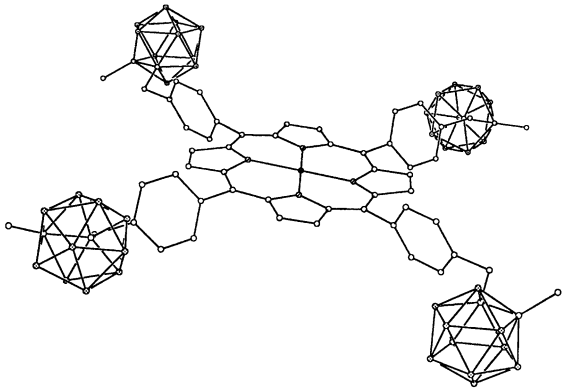


Figure 1. The molecular structure of Zn(II)-porphyrin **6**[33] illustrating the four carborane–porphyrin linkages and the waved conformation of the porphyrin macrocycle (hydrogen atoms not shown).

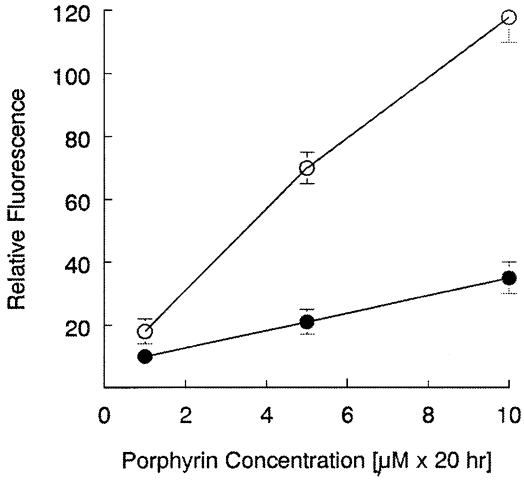


Figure 2. Concentration–dependent uptake of carboranylporphyrins **5** (open circles) and **7** (filled circles) by rat 9L gliosarcoma cells.

Table 1. IC_{50} concentrations (μM) obtained after 24 h porphyrin exposure to cells

Porphyrin	IC_{50} (μM) in 9L and U-373MG Cells	IC_{50} (μM) in B16 cells
5	150	>250
7	180	>250
12	150	>250
14	185	>250

caused cytotoxicity following 2 h ($IC_{50} = 50\,\mu M$) and 24 h ($IC_{50} = 1.5\,\mu M$) time exposures. As observed in the dark toxicity experiments, the corresponding Zn(II) complex **7** was approximately 20% less phototoxic for all cell types, and the mouse B16 cells were more photoresistant than were the rat and human cells. Plasma membrane blebbing and nuclear condensation could be seen by phase microscopy within two hours following irradiation of the cells, suggesting the onset of apoptosis.

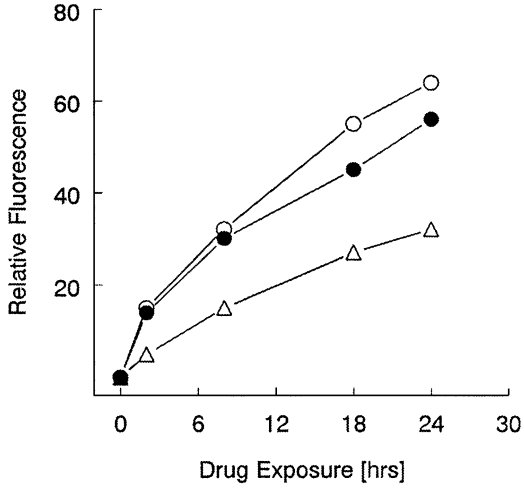


Figure 3. Time-dependent uptake of carboranylporphyrin **5** (5 μM) by rat 9L cells (open circles), human U-373MG cells (closed circles) and mouse B16 cells (open triangles).

Cellular uptake

The concentration-dependent uptake was investigated in cells exposed to 1, 5, and 10 μM of each porphyrin for 24 h. The concentration of intracellular-bound porphyrin was determined by chemical extraction of washed cell monolayers, followed by spectrophotometric and/or ICP-MS determinations. The uptake values for 9L and U-373MG cells were very similar and exceeded that of B16 cells. The porphyrin accumulation invariably increased with increasing exogenous drug concentration and the uptake of *nido*-carboranylporphyrins **5** and **12** was approximately four-times greater than that of the corresponding Zn(II) complexes **7** and **14**. A comparison of the concentration-dependent uptake of porphyrin **5** and the corresponding zinc complex **7** by 9L cells is depicted in Figure 2.

The uptake of carboranylporphyrins by log phase cells was also shown to be time-dependent, as is illustrated in Figure 3 for porphyrin **5**. Cells exposed to 5 μM drug concentrations contained increasing amounts of extractable porphyrin over the 24 h uptake time period examined in these studies. As previously observed for the concentration-dependent uptake, 9L and U-373MG cells had similar time-dependent uptake levels, while B16 cell cultures consistently accumulated 60–70% less drug on a per cell basis. Cell-bound porphyrin that could not be removed using Hanks balanced salt solution (HBSS) washes was detectable as early as 1 h after introducing the drug to the cell culture. When rat 9L cells were exposed to 10 μM concentrations of free-base porphyrins **5** and **12** for 24 h, intracellular boron levels of 53.5 ± 5.0 and 54.5 ± 3.3 μg of boron per billion cells (or per gram of wet tissue) were achieved, respectively, for **5** and **12**. The Zn(II) complexes consistently produced up to 4 times lower intracellular boron levels.

The retention of *nido*-carboranylporphyrins in cells was determined by placing washed cells in drug-free medium, following exposure to 5 μM concentration of porphyrin for 24 h. Cells followed for 8 h in drug-free medium showed drug losses of less than 20% over this time period (Fig. 4). Figure 4 also shows the similarity in uptake and

retention by carboranylporphyrin **5** and its *meta*-regioisomer **12**, and the dramatic reduction in uptake of the zinc complex **14**.

In order to study the influence of plasma proteins on cellular uptake, porphyrin **5** was pre-incubated with dilutions of normal rat or human plasma for 15 min at 37 °C, prior to administration to rat 9L or human U-373MG cells growing in low (0.5%) or high (5%) serum supplemented culture medium. Figure 5 shows that uptake of carboranylporphyrin **5** was reduced in human U-373MG cells as the concentration of pre-incubation human plasma was increased. A similar inhibitory effect on uptake was seen in rat 9L cells when the carboranylporphyrins were pre-incubated in dilutions of rat plasma (data not shown). The inhibitory effect of plasma on porphyrin uptake or cell binding was obscured when the uptake was examined in cells growing in medium supplemented with 5% fetal bovine serum (Fig. 5).

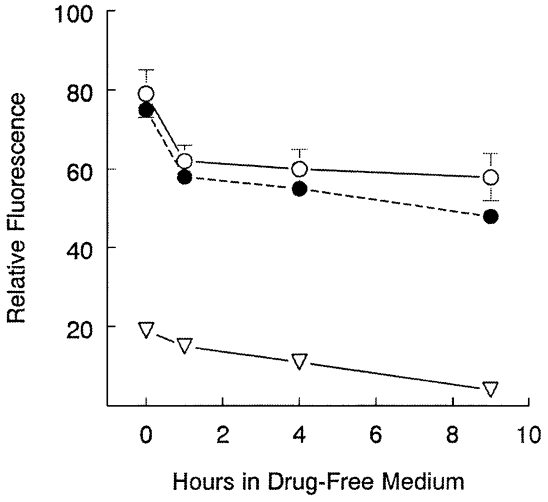


Figure 4. Retention of carboranylporphyrin **5** (open circles), **12** (closed circles), and **14** (open triangles) at concentrations of 5 μM, by rat 9L cells after 24 h uptake time.

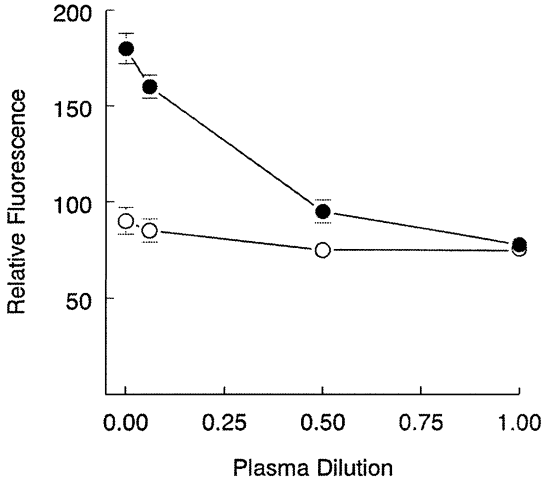


Figure 5. Influence of human plasma concentration on the uptake of carboranylporphyrin **5** by human U-373MG cells in 5% fetal bovine serum (open circles) and 0.5% serum (closed circles).

Intracellular localization

Confocal fluorescence microscopy was used to examine the intracellular localization of *nido*-carboranylporphyrin **5** in live cells. Rat 9L tumor cells and the human normal keratinocyte line HaCaT were used in these studies. HaCaT cells were included in these particular experiments because they adhere and spread nicely on glass cover slips, thus facilitating the imaging process. Cells exposed to 2–20 μM concentrations of porphyrin **5** for 24 h were examined for intracellular fluorescence. Both the 9L and HaCaT cells showed similar intracellular fluorescent pattern, and in both cases, 100% of the cells were labeled. As can be seen in Figure 6, the punctuate fluorescence was predominately perinuclear, with many cells having an additional local area of concentration that appeared to be adjacent to the nuclear membrane. Preliminary co-localization experiments using the organelle-specific fluorescent probes lyso tracker green, mito tracker green, BODIPY C5-ceramide, rhodamine B hexyl ester and Hoechst 33342, suggest that porphyrin **5** localizes preferentially in the cell lysosomes. No fluorescent signal was detectable in the plasma membranes of isolated individual cells nor in the intercellular junctions of confluent cells.

Discussion

Two carboranyl-containing regioisomeric porphyrins (*para*- and *meta*-substituted on the *meso*-phenyl rings) and their zinc(II) complexes were synthesized in order to study and compare their biological properties. All porphyrins prepared possess chemically stable carbon-carbon linkages between the *meso*-phenyl substituents and the carborane moieties, and 36–40 atoms of boron per molecule (26–32% boron by weight). Symmetrical *meso*-tetraphenylporphyrin derivatives are readily obtained, usually in good yields and high purity, by

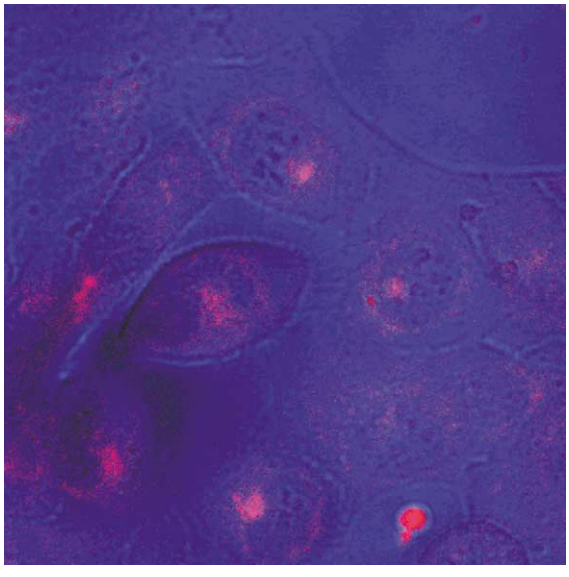


Figure 6. Intracellular localization of carboranylporphyrin **5** in HaCaT keratinocytes determined by confocal fluorescence microscopy.

acid-catalyzed tetramerization of a substituted benzaldehyde (such as **3** and **10**) with pyrrole.[35] We rationalized that the effect of the distribution of the carborane cages at the porphyrin periphery and the presence of a chelated zinc ion could potentially influence the cytotoxicity, cell uptake and retention, and the target subcellular organelle of carboranylporphyrins. We have recently discovered that the acid-base, aggregation and some spectroscopic properties of this type of compounds are substantially determined by the position of the carborane cages at the periphery of the porphyrin macrocycle.[33,36] In Figure 1, the non-planar waved conformation of zinc porphyrin **6**[33] and the four methylene bridges between the porphyrin and carborane moieties are shown.

The highly hydrophobic character of the *ortho*-carborane cages of porphyrins **4**, **6**, **11** and **13** renders complete insolubility of these compounds in aqueous solutions. To achieve water-solubility, the *nido*-carboranyl derivatives **5**, **7**, **12**, and **14** were prepared, by basic degradation of the *ortho*-carborane cages. Although tetra-anionic in nature, these tetra(*nido*-carboranyl)porphyrins display amphiphilic character, that is, they are soluble in dimethylsulfoxide (DMSO), acetone, methanol, and in water at physiological pH and at concentrations up to 1.7 mM. The amphiphilic properties are highly desirable in promising new BNCT agents, for both solubility in blood and for crossing lipophilic membranes. All tetra(*nido*-carboranyl)porphyrins display higher hydrophobicities than, for example, PP-IX and $TPP(CO_2H)_4$, as evaluated by their distributions between 1-octanol and a buffered aqueous solution [in order of decreasing hydrophobicity **5** > **12** > **7** > **14** > PP-IX > $TPP(CO_2H)_4$]. The zinc(II) complexes **7** and **14** were found to have lower hydrophobic characters than the corresponding free-bases **5** and **12**, probably because of the higher tendency of the metal-free carboranylporphyrins to form aggregates at physiological pH.[36] The amphiphilic character of the *nido*-carboranylporphyrins probably accounts for their ready uptake by cells (*vide infra*).

The preliminary in vitro evaluation of the tetra-anionic *nido*-carboranylporphyrins **5**, **7**, **12**, and **14** was accomplished using well-established cell lines in culture. The results obtained from these studies provide an insight into the biological characteristics of these porphyrins and help to predict and possibly interpret future in vivo studies. All *nido*-carboranylporphyrins are soluble in aqueous media, relatively nontoxic when applied directly to mitotically active cells, and are readily taken up and retained by stationary phase and proliferating cells. Solubility and toxicity are key limiting factors that often prevent newly developed boron-containing compounds from becoming practically useful in a clinical setting, in view of the high boron concentration requirement in BNCT for effective cell killing ($\sim 10^9$ ^{10}B atoms/cell). While cell culture tests have definite limitations and cannot substitute for the complex physiology of in vivo models, it is encouraging that a variety of animal cell types were not adversely affected by high concentrations and prolonged exposure in the dark to each of the compounds described (Table 1). The *nido*-carboranylporphyrins used in this study are among the least toxic in the dark to cells in vitro of all carborane-containing porphyrins reported to date in literature.[19,37] In the presence of light all porphyrins studied were significantly more toxic, as is characteristic of many porphyrin-type macrocycles; this light-induced toxicity is probably caused by the production of singlet oxygen, which is a powerful oxidant and has a very short diffusion range in cells (approximately 0.1 μm). These results also suggest that the subcellular sites of localization of these porphyrins are highly appropriate for eliciting a cytotoxic response. High concentrations of the zinc(II) complexes **7** and **14** appear to be slightly better tolerated by cells but this may be a reflection of the decreased cellular uptake of these derivatives, possibly due to their decreased hydrophobicity compared with the free-base porphyrins. For both mouse B16 and rat 9L cells, a 24 h exposure time represents more than a complete cell cycle (cycle time is ca. 18 h for B16 and 9L cells under culture conditions in these experiments). It is worthwhile to underline that the characteristic photosensitizing properties and fluorescence of porphyrin macrocycles are retained in carborane-containing porphyrins, as has been previously observed.[17,21,24,26,29] This presents the possibility that these versatile compounds could also have applications as sensitizers for PDT and/or fluorescent based tissue localization and quantification. In fact, a carborane-containing porphyrin, BOPP, has shown promise as PDT photosensitizer for the treatment of malignant gliomas.[24,38]

Mouse B16 cells consistently accumulated less porphyrin than the other cell types when tested under identical culture conditions, as can be seen in Figure 3. This suggests that uptake/retention as measured in these studies was not due merely to non-specific association of porphyrins with any cellular entity. The molecular basis for the observed preferential accumulation in 9L and U-373MG cells over B16 cells is not yet known. These cells are similar in size and proliferation rate, so these parameters apparently are not influential. Further insight into the basis for cell selectivity could be gained by testing each compound against a panel of related cell types with known qualitative or quantitative differences in one significant feature such as density of low density lipoprotein receptors, or endocytic activity.

All carboranylporphyrins studied show similar concentration-dependent accumulation by cells, with suppression of uptake by increasingly higher concentrations (1–10%) of fetal bovine serum.[39] This observation has been previously reported for carboranyl-containing porphyrins,[19] and probably reflects the ability of these compounds to bind to proteins within the serum, thus inhibiting them from subsequent uptake by cells. When solutions of protein-free carboranylporphyrins were incubated in dilutions of unfractionated plasma (human or rat), their subsequent uptake by cells was reduced (Fig. 5). This suggests that these porphyrins associate with as yet unidentified plasma component(s) that inhibit some step in the binding and eventual uptake of porphyrins by cells in vitro. When this experiment was performed using cells growing in high serum media, the plasma pre-incubation effect was not observed because

the serum itself was sufficiently concentrated to reduce porphyrin uptake to a minimum.

The Zn(II) complexes **7** and **14** were accumulated intracellularly to a much lesser extent than the metal-free counterparts (Figs 2 and 4); this effect may be due to the lower hydrophobicity of the Zn(II) complexes compared with the metal-free porphyrins, as a result of the higher aggregation tendency of the later compounds.[36] The increase cellular uptake with increasing hydrophobic character of *nido*-carboranylporphyrins has been previously reported for free-base macrocycles, although the reverse relationship was observed for the corresponding zinc complexes.[19] In addition, and contrary to our observations, the zinc porphyrins studied were found to be taken up by V79 hamster lung cells to a greater extent than the free-bases, although displaying lower hydrophobic character.

Although significantly different physicochemical properties have been recently observed for the *para*- and *meta*-regioisomers of *nido*-carboranylporphyrins,[33,36] the in vitro uptake and efflux properties of porphyrins **5** and **12** are very similar. The slightly more rapid efflux observed for porphyrin **12** compared with **5** (Fig. 4) is not statistically significant, and these experiments are currently being extended under a variety of conditions to more clearly define biological differences mediated by the distribution of the *nido*-carboranyl groups at the porphyrin periphery.

Since all the *nido*-carboranylporphyrins studied display very low cytotoxicities (Table 1), a large dose of porphyrin can in principle be administered to cells in culture and subsequently to animals in order to achieve high concentrations of boron in tumors. Indeed, high concentrations of boron were achieved for the free-base porphyrins **5** and **12** using 10 μM concentrations (>49 μg of boron/g tissue after a 24 h period), which are well below the IC_{50} values. Therefore both of these compounds could potentially provide therapeutic boron levels for effective boron neutron capture therapy. The high retention observed for these *nido*-carboranylporphyrins (Fig. 4) and other related compounds suggests significant interactions of these porphyrins with cellular constituents. The intracellular boron distribution plays an important role in BNCT effectiveness. Tumor cell nuclei, and in particular DNA, are the most desirable targets for the high LET particles generated in the boron nuclear reaction.[40] A lower cellular boron concentration is assumed to be required if the boron-containing drug localizes inside or in close proximity of the cell nucleus. By fluorescence microscopy *nido*-carboranylporphyrin **5** was found in the region surrounding the cell nucleus of the rat and human cell types examined, with a special area of concentration adjacent to the nuclear membrane that appeared at first to be Golgi-like (Fig. 6). However, co-localization experiments using organelle-specific fluorescent probes showed that the Golgi apparatus was not a site for localization of **5**, and that the lysosomes were the preferential sites of localization for this porphyrin. This is not a surprising result since negatively-charged porphyrins tend to target the cell-lysosomes, which are characterized by a low internal pH. Anionic carborane-containing porphyrins (BOPP and VCDP) have been reported to mainly localize in the lysosomes and endoplasmic reticulum of 9L cells.[37] Both of these compounds were also found in mitochondria, possibly due to their binding to the outer membrane benzodiazepine receptors, and to a smaller extent in the cell nuclei. In a human glioma cell line (SF-767) BOPP was found mainly in the cell-lysosomes.[30]

Conclusions

Nido-carboranylporphyrins display several in vitro characteristics that make them highly suitable for continued evaluation as potential new BNCT agents, namely low cytotoxicity, substantial uptake and retention by cells in culture, affinity for serum components and favorable intracellular sites of localization. These negatively charged compounds of amphiphilic character are highly soluble in aqueous solutions while still possessing remarkable cell membrane penetration capabilities. The four tetra(*nido*-carboranyl)porphyrins studied all displayed low cytotoxicities, with IC_{50} values higher than 150 μM in all cell lines. Low toxicity is perhaps the most important characteristic of a promising new boron-delivering agent, since it allows the administration of a sufficiently high drug dose to achieve an effective therapeutic effect.

The preferential subcellular sites of localization of a metal-free tetra(*nido*-carboranyl)porphyrin were found to be in close proximity to the nuclear membrane and in the cell-lysosomes; if a similar situation is observed in vivo, effective tumor cell destruction can be achieved by release of the high LET particles intracellularly, in the proximity of the cell nucleus.

The in vitro properties of the metal-free carboranylporphyrins seem to be little affected by the distribution of the carborane residues about the porphyrin macrocycle (*para*- vs *meta*-regioisomers). In contrast, however, the presence of a central chelated zinc ion has a significant effect on cell uptake and cytotoxicity, possibly as a result of the lower hydrophobic character and tendency for aggregation of the zinc complexes. Both metal-free carboranylporphyrins **5** and **12** are the most promising candidates for use as BNCT agents of the four compounds studied, since therapeutic amounts of boron were achieved in cells in vitro, using porphyrin concentrations well below toxic level.

The *nido*-carborane-containing porphyrins studied were found to retain their characteristic fluorescence and photosensitizing properties, typical of porphyrin macrocycles. Therefore, tumor-selective carboranyl-porphyrins of low toxicity could also have potential application as sensitizers for the PDT treatment of cancer. The high stability of the carbon-carbon linkages between the porphyrin macrocycles and the carborane cages will ensure that in vivo cleavage, leading to release of free-boron, is highly improbable with these compounds. The direct in vitro evaluation of potential new BNCT agents

is advantageous for initial compound screening and inter-comparison since it is both time and cost effective and uses only small amounts of compounds; these studies facilitate the design and development of new boron-delivery agents for effective BNCT. The in vivo toxicity and biodistribution properties of *nido*-carboranylporphyrins are currently being evaluated in our laboratories.

Experimental

Melting points were measured on a Thomas/Bristoline microscopic hot stage apparatus and were uncorrected. Silica gel 60 (70–230 and 230–400 mesh, Merck) or neutral alumina (Merck; usually Brockmann Grade III, i.e., deactivated with 6% water) were used for column chromatography. Analytical thin-layer chromatography (TLC) was performed using Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick). Reactions were monitored by TLC and spectrophotometry. ^{1}H NMR spectra were obtained in either deuterochloroform or acetone-d_6 solution, using a Brucker Inova 400 MHz spectrometer; chemical shifts are expressed in ppm relative to chloroform (7.26 ppm) and/or TMS (0 ppm). Unless otherwise stated, electronic absorption spectra were measured in dichloromethane solution using a Hewlett-Packard 8450A spectrophotometer. Mass spectra were obtained at the Mass Spectrometry Facility, University of California, San Francisco, CA, USA. The bromomethylbromobenzenes **1** and **8** were purchased (Aldrich) and used without further purification. Trifluoroacetic acid (TFA) and *n*-butyllithium (1.6 M in hexanes) were purchased from Fluka, and 1-methyl-*o*-carborane from Dexsil Corporation (Hamden, CT, USA) and used without further purification. All solvents were dried and purified according to literature procedures.[41]

Cell culture RPMI 1640 medium and HBSS were obtained from Mediatech, Inc. (Herndon, VA, USA), fetal bovine serum from Life Technologies, Inc. (Rockville, MD), Alamar Blue from AccuMed, Inc. (Chicago, IL, USA), HEPES [*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)] from Sigma Chemicals (St. Louis, MO, USA) and lyso tracker green, mito tracker green, BODIPY FL C5-ceramide, rhodamine B hexyl ester chloride and Hoechst 33342 were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Spectrofluorometric analysis was performed using a Perkin-Elmer LS-5B luminescence spectrometer. Microscopy was performed on a Nikon Eclipse TE300 inverted phase microscope and on a Leica DM IRBE confocal microscope with TCS SP multiband confocal imaging spectrophotometer equipped with a krypton laser (568 nm light). A Hewlett-Packard HP 4500 ICP-MS (Yokohama Analytical Systems, Tokyo, Japan) equipped with a cross-flow nebulizer and polypropylene spray chamber was used for analytical boron determinations.

[4-(1-Methyl-*o*-carboranyl)methyl]bromobenzene (2). A two-necked round bottom flask containing 1-methyl-*o*-carborane (5.00 g, 31.65 mmol) in dry DME (150 mL) was cooled down to 0 °C under Argon. *n*-BuLi (20.0 mL, 1.6 M in hexane) was added dropwise and the resulting mixture was stirred at 0 °C for 30 min. A solution of 4-(bromomethyl)bromobenzene (7.91 g, 31.65 mmol) in dry DME (15 mL) was added dropwise. After stirring at 0 °C for 10 min, the final reaction mixture was warmed to room temperature and subsequently refluxed for 10 h under argon. The solvent was then removed under vacuum and the crude solid obtained was purified by recrystallization from dichloromethane/methanol to give the title compound (7.80 g, 75.4% yield) as white crystals, mp 127–128 °C. ^{1}H NMR ($CDCl_3$) δ 1.3–3.0 (br, 10H, BH), 2.15 (s, 3H, CH_3), 3.41 (s, 2H, CH_2), 7.06 (d, 2H, ArH, J = 8.1 Hz), 7.48 (d, 2H, ArH, J = 8.1 Hz). MS (EI) m/e 327.1 (M^+).

[4-(1-Methyl-*o*-carboranyl)methyl]benzaldehyde (3). A solution of compound **2** (4.00 g, 12.23 mmol) in THF (150 mL) under argon was cooled to −78 °C. *n*-BuLi (7.6 mL, 1.6 M in hexane) was added dropwise while maintaining the temperature at −78 °C. The reaction mixture was stirred for 30 min at −78 °C before dry DMF (5.0 mL, 64.6 mmol) was slowly added. The final mixture was stirred at −78 °C for 15 min and then warmed slowly to room temperature. A 2 N HCl solution (150 mL) was added and the reaction mixture stirred for 2 h at room temperature. The solution was then reduced to a volume of 200 mL and extracted into CH_2Cl_2 (4×50 mL). The organic extracts were washed once with aqueous saturated $NaHCO_3$, once with water and dried over anhydrous Na_2SO_4. After removal of the solvent under vacuum, the oily residue was purified by column chromatography on silica gel (CH_2Cl_2/petroleum ether, 1:1), yielding the title compound (2.12 g, 62.2% yield) as a white solid, mp 96–97 °C. ^{1}H NMR ($CDCl_3$) δ 1.5–3.0 (br, 10H, BH), 2.19 (s, 3H, CH_3), 3.54 (s, 2H, CH_2), 7.38 (d, 2H, ArH, J = 8.0 Hz), 7.89 (d, 2H, ArH, J = 8.0 Hz), 10.04 (s, 1H, CHO). MS (EI) m/e 276.2 (M^+).

***meso*-Tetra[4-(1-methyl-*o*-carboranyl)methylphenyl]porphyrin (4).** A solution of aldehyde **3** (1.16 g, 4.19 mmol) and freshly distilled pyrrole (0.30 mL, 4.32 mmol) in dry CH_2Cl_2 (420 mL) was purged with argon for 15 min. TFA (0.25 mL, 3.15 mmol) was added to the solution and the mixture was stirred at room temperature under argon for 20 h (complete disappearance of starting compound **3** monitored by TLC). After addition of *p*-chloranil (0.788 g, 3.14 mmol) the reaction mixture was stirred at room temperature for 2 h. The solution was concentrated under vacuum to 200 mL, then washed once with aqueous saturated $NaHCO_3$, and once with water before being dried over anhydrous Na_2SO_4. The residue obtained after removal of the solvent under vacuum was purified by column chromatography on silica gel (CH_2Cl_2/petroleum ether 1:1) and the fastest running porphyrin fraction was collected and recrystallized from CH_2Cl_2/methanol, yielding 0.375 g (32.1% yield) of the title compound as purple crystals, mp > 300 °C. ^{1}H NMR ($CDCl_3$) δ −2.80 (br, 2H, NH), 1.6–3.1 (br, 40H, BH), 2.34 (s, 12H, CH_3), 3.81 (s, 8H, CH_2), 7.59 (d, 8H, ArH, J = 8.0 Hz), 8.20 (d, 8H, ArH, J = 8.0 Hz), 8.85 (s, 8H, β-H). ^{1}H NMR ($CDCl_3$/TFA-*d*)

δ −1.08 (br, 4H, NH), 1.6-3.0 (br, 40H, BH), 2.38 (s, 12H, CH_3), 3.89 (s, 8H, CH_2), 7.87 (d, 8H, ArH, $J=7.8$ Hz), 8.54 (d, 8H, ArH, $J=7.8$ Hz), 8.73 (s, 8H, β-H). UV–vis ($CHCl_3$) λ_{max} 417 nm (ε 444,700), 514 (15,400), 548 (7200), 588 (5100), 644 (3800). MS (MALDI) *m/e* 1296.0 (M^+).

***meso*-Tetra[4-(1-methyl-*nido*-carboranyl)methylphenyl]-porphyrin tetrapotassium salt (5).** Porphyrin **4** (0.050 g, 0.0386 mmol) was dissolved in a 3:1 mixture of pyridine and piperidine (4.0 mL), and stirred at room temperature in the dark for 36 h, under argon. The solvent was completely removed under vacuum, the residue redissolved in 40% aqueous acetone and passed slowly through a Dowex 50WX2-100 resin in the potassium form. The porphyrin fraction was collected, dried under vacuum, redissolved in 70% aqueous acetone and again passed through the ion-exchange resin. After removal of the solvent under vacuum, the tetraanionic porphyrin was twice recrystallized from methanol/diethyl ether, yielding 0.051 g (93.8% yield) of the title compound, mp > 300 °C. ^{1}H NMR (CD_3COCD_3) δ −2.70 (s, 2H, NH), −2.45 to −1.90 (br, 4H, BH), 0.9–2.4 (br, 32H, BH), 1.59 (s, 12H, CH_3), 3.50 (s, 8H, CH_2), 7.81 (d, 8H, ArH, $J=8.0$ Hz), 8.08 (d, 8H, ArH, $J=8.0$ Hz), 8.90 (s, 8H, β-H). UV–vis (acetone) λ_{max} 420 nm (ε 349,700), 516 (13,600), 554 (12,400), 594 (4100), 650 (6000). MS (MALDI) *m/e* 1408.60 (M^+).

Zinc(II) *meso*-tetra[4-(1-methyl-*o*-carborane)methylphenyl] porphyrin (6). To a solution of porphyrin **4** (0.150 g, 0.110 mmol) in dichloromethane (150 mL), THF (10 mL), and pyridine (0.5 mL) was added $ZnCl_2.2H_2O$ (0.075 g, 0.435 mmol), and the final mixture was stirred at room temperature under argon overnight. The mixture was then washed once with water, dried over anhydrous Na_2SO_4, and the solvent evaporated under vacuum. The residue was purified by column chromatography (dichloromethane/petroleum ether 1:1.5), the pink colored fraction collected and recrystallized from dichloromethane/methanol, to give 0.135 g (86.0% yield) of the title compound as purple crystals, mp > 300 °C. ^{1}H NMR ($CDCl_3$) δ 1.6–3.0 (br, 40H, BH), 2.34 (s, 12H, CH_3), 3.82 (s, 8H, CH_2), 7.58 (d, 8H, ArH, $J=7.8$ Hz), 8.20 (d, 8H, ArH, $J=7.8$ Hz), 8.96 (s, 8H, β-H). UV–vis ($CHCl_3$) λ_{max} 424 nm (ε 577,000), 554 (20,100), 596 (6400). MS (MALDI) *m/e* 1358.60 (M^+).

Zinc(II) *meso*-tetra[4-(1-methyl-*nido*-carboranyl)methylphenyl]porphyrin tetrapotassium salt (7). The Zn(II) complex **6** (0.075 g, 0.055 mmol) was dissolved in a 3:1 mixture of pyridine and piperidine (4.0 mL), and stirred at room temperature in the dark for 36 h, under argon. The solvent was removed under vacuum, the residue was redissolved in 40% aqueous acetone and passed slowly through a Dowex 50W2-100 resin in the potassium form. The porphyrin fraction was collected, dried under vacuum, redissolved in 70% aqueous acetone and again passed through the ion-exchange resin. After removal of the solvent under vacuum, the tetraanionic porphyrin was recrystallized from methanol/diethyl ether, yielding 0.078 g (96.1% yield) of the title compound, mp > 300 °C. ^{1}H NMR (CD_3COCD_3) δ −2.48 to −1.95 (br, 4H, BH), 0.9–2.4 (br, 32H, BH), 1.59 (s, 12H, CH_3), 3.50 (s, 8H, CH_2), 7.77 (d, 8H, ArH, $J=8.0$ Hz), 8.11 (d, 8H, ArH, $J=8.0$ Hz), 8.92 (s, 8H, β-H). UV–vis (acetone) λ_{max} 422 nm (ε 479,000), 554 (13,900), 596 (6,600). MS (MALDI) *m/e* 1473.90 ($M^+ + H$).

[3-(1-Methyl-*o*-carboranyl)methyl]bromobenzene (9). A two-necked round bottom flask containing 1-methyl-*o*-carborane (3.00 g, 18.99 > mmol) in dry THF (150 mL) was cooled to 0 °C under argon. *n*-BuLi (12.0 mL, 1.6 M in hexane) was added dropwise and the resulting mixture was stirred at 0 °C and then cooled to −10 °C. A solution of anhydrous LiI (0.350 g, 2.61 mmol) in THF (2.5 mL) was added, followed by a solution of 3-(bromomethyl)bromobenzene (5.00 g, 20.00 mmol) in THF (10 mL). After stirring at −10 °C for 15 min, the reaction mixture was warmed to room temperature and stirred for 12 h under argon. The reaction mixture was then washed with water (2×25 mL), extracted with diethyl ether (3×25 mL) and dried over anhydrous Na_2SO_4. The solvent was then removed under vacuum and the crude solid obtained was purified by column chromatography (silica gel, dichloromethane/petroleum ether, 1:9) to give the title compound (4.25 g, 68.5% yield), mp 77–78 °C. ^{1}H NMR ($CDCl_3$) δ 1.3–3.1 (br, 10H, BH), 2.16 (s, 3H, CH_3), 3.42 (s, 2H, CH_2), 7.13 (d, 1H, ArH, $J=7.8$ Hz), 7.23 (t, 1H, ArH, $J=7.8$ Hz), 7.33 (s, 1H, ArH), 7.47 (d, 1H, ArH, $J=7.8$ Hz). MS (EI) *m/e* 327.2 (M^+).

[3-(1-Methyl-*o*-carboranyl)methyl]benzaldehyde (10). A solution of compound **9** (1.00 g, 3.06 mmol) in THF (25 mL) under argon was cooled to −78 °C (acetone/dry ice bath). *n*-BuLi (2.0 mL, 1.6 M in hexane) was added dropwise while maintaining the temperature at −78 °C. The reaction mixture was stirred for 30 min at −78 °C before dry DMF (1.0 mL, 17.5 mmol) was slowly added. The mixture was then stirred at −78 °C for 15 min and then warmed up slowly to room temperature. A 2 N HCl solution (25 mL) was added and the reaction mixture was stirred for 2 h at room temperature. The solution was then diluted with water and extracted into dichloromethane (4×50 mL). The organic extracts were washed once with aqueous saturated $NaHCO_3$, once with water and then dried over anhydrous Na_2SO_4. After removal of the solvent under vacuum, the oily residue was purified by column chromatography on silica gel (dichloromethane/petroleum ether, 1:1), yielding the title compound (0.668 g, 79.1% yield) as a white solid, mp 93–94 °C. ^{1}H NMR ($CDCl_3$) δ 1.4–3.1 (br, 10H, BH), 2.19 (s, 3H, CH_3), 3.55 (s, 2H, CH_2), 7.48 (d, 1H, ArH, $J=7.8$ Hz), 7.56 (t, 1H, ArH, $J=7.8$ Hz), 7.70 (s, 1H, ArH), 7.85 (d, 1H, ArH, $J=7.8$ Hz), 10. 04 (s, 1H, CHO). MS (EI) *m/e* 276.2 (M^+).

***meso*-Tetra[3-(1-methyl-o-carboranyl)methylphenyl]porphyrin (11).** A solution of aldehyde **10** (0.660 g, 2.39 mmol) and freshly distilled pyrrole (0.18 mL, 2.59 mmol) in dry dichloromethane (240 mL) was purged with argon for 15 min. TFA (0.15 mL, 1.89 mmol) was added to the solution and the final mixture was stirred at room temperature under argon for 18 h. After addition of *p*-chloranil (0.440 g, 1.77 mmol) the reaction mixture was stirred at room

temperature for 3 h. The organic solution was washed once with aqueous saturated $NaHCO_3$, and once with water before being dried over anhydrous Na_2SO_4. The residue obtained after removal of the solvent under vacuum was purified by column chromatography on silica gel (dichloromethane/petroleum ether, 1:1) and the porphyrin fraction was collected and recrystallized from dichloromethane/methanol, yielding 0.252 g (33.0% yield) of the title compound as purple crystals, mp 276–279 °C. ^{1}H NMR ($CDCl_3$) δ −2.84 (br, 2H, NH), 1.5–3.0 (br, 40H, BH), 2.20 (s, 12H, CH_3), 3.74 (s, 8H, CH_2), 7.62 (d, 4H, ArH), 7.74 (d, 4H, ArH), 8.05 (d, 4H, ArH), 8.18 (d, 4H, ArH), 8.84 (s, 8H, β-H). UV–vis ($CHCl_3$) λ_{max} 417 nm (497,500), 514 (19,100), 549 (8800), 588 (6100), 645 (4700). MS (MALDI) *m/e* 1295.7 (M^+).

***meso*-Tetra[3-(1-methyl-nido-carboranyl)methylphenyl]-porphyrin tetrapotassium salt (12).** Porphyrin **11** (0.049 g, 0.0378 mmol) was dissolved in a 3:1 mixture of pyridine and piperidine (4.0 mL), and stirred at room temperature in the dark for 36 h, under argon. The solvent was completely removed under vacuum, the residue redissolved in 40% aqueous acetone and passed slowly through a Dowex 50WX2-100 resin in the potassium form. The porphyrin fraction was collected, dried under vacuum, redissolved in 70% aqueous acetone and again passed through the ion-exchange resin. After removal of the solvent under vacuum, the tetraanionic porphyrin was recrystallized from methanol/diethyl ether, yielding 0.050 g (94.0% yield) of the title compound, mp > 300 °C. ^{1}H NMR (CD_3COCD_3) δ −2.70 (br, 2H, NH), −2.54 to −2.00 (br, 4H, BH), 0.9–2.3 (br, 32H, BH), 1.58 (s, 12H, CH_3), 3.48 (s, 8H, CH_2), 7.70 (m, 4H, ArH), 7.94 (m, 8H, ArH), 8.22 (m, 4H, ArH), 8.97 (s, 8H, β-H). UV–vis (acetone) λ_{max} 414 nm (ε 358,200), 512 (18,600), 546 (11,000), 591 (5700), 648 (10,500). MS (MALDI) *m/e* 1407.20 (M^+).

Zinc(II) *meso*-tetra[3-(1-methyl-*o*-carboranyl)methylphenyl] porphyrin (13). To a solution of porphyrin **11** (0.040 g, 0.0308 mmol) in dichloromethane (25 mL), THF (2 mL), and pyridine (0.2 mL) was added $ZnCl_2$ (0.020 g, 0.146 mmol), and the mixture was stirred at room temperature under argon overnight. The mixture was then washed once with water, dried over anhydrous Na_2SO_4, and the solvent evaporated under vacuum. The residue was purified by column chromatography on silica gel (dichloromethane/petroleum ether, 1:1.5), the pink fraction collected and recrystallized from dichloromethane/methanol, to give 0.038 g (90.5% yield) of the title compound as purple crystals, mp 190–195 °C; ^{1}H NMR ($CDCl_3$) δ 1.6–3.0 (br, 40H, BH), 2.21 (s, 12H, CH_3), 3.76 (s, 8H, CH_2), 7.63 (d, 4H, ArH), 7.75 (m, 4H, ArH), 8.07 (d, 4H, ArH), 8.18 (m, 4H, ArH), 8.93 and 8.94 (s, 4H each, β-H). UV–vis ($CHCl_3$) λ_{max} 422 nm (ε 596,300), 552 (22,600), 594 (6800). MS (MALDI) *m/e* 1359.5 (M^+).

Zinc(II) *meso*-tetra[3-(1-methyl-*nido*-carboranyl)methylphenyl]porphyrin tetrapotassium salt (14). The zinc complex **13** (0.025 g, 0.0183 mmol) was dissolved in a 3:1 mixture of pyridine and piperidine (3 mL), and stirred at room temperature in the dark for 36 h, under argon. The solvent was completely removed under vacuum, the residue redissolved in 40% aqueous acetone and passed slowly through a Dowex 50WX2-100 resin in the potassium form. The porphyrin fraction was collected, dried under vacuum, redissolved in 70% aqueous acetone and again passed through the ion-exchange resin. After removal of the solvent under vacuum, the tetraanionic porphyrin was recrystallized from methanol/diethyl ether, yielding 0.026 g (95.9% yield) of the title compound, mp > 300 °C. ^{1}H NMR (CD_3COCD_3) δ −2.6 to −2.1 (br, 4H, BH), 0.9–2.3 (br, 32H, BH), 1.56 (s, 12H, CH_3), 3.44 (s, 8H, CH_2), 7.66 (m, 4H, ArH), 7.93 (m, 8H, ArH), 8.16 (m, 4H, ArH), 8.90 and 8.95 (d, 4H each, β-H). UV–vis (acetone) λ_{max} 420 nm (ε 578,600), 553 (16,500), 594 (8500). MS (MALDI) *m/e* 1473.30 ($M + H^+$).

1-Octanol/aqueous buffer distribution coefficients. The distribution coefficients were determined at room temperature, by adding 0.10 mL of porphyrin stock solution to phase separation tubes containing 2.0 mL of 0.05 mM phosphate buffered solution (pH 7.4) and 2.0 mL of 1-octanol. The tubes were vortexed for 1 min and then the two phases were allowed to separate by gravity. An aliquot from each layer (0.10 mL) was removed for spectrofluorometric analysis, by dilution with 2.9 mL of methanol/HEPES 10 mM, in 1:1 ratio.

Cell culture

Established cell lines (rat 9L gliosarcoma and mouse B16 melanoma, human U-373MG glioblastoma) were maintained as monolayer cultures in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Phenol red and antibiotics were not included in the media. After cell lines had undergone 20 passages they were replaced by low passage cells from frozen parental stocks.

Cytotoxicity

To assess the cytostatic and cytotoxic effects of the carboranylporphyrins, log phase cells were trypsinized, counted and seeded into 96-well plates at $1–3\times10^3$ cells per well. Cells were allowed to settle and attach for 24–48 h, then the porphyrins were added to triplicate wells in 2-fold serial dilutions from 250 to 1.0 μM for dark toxicity or from 10 to 0.04 μM for light-mediated toxicity. Cells were exposed to the drugs for a brief (2 h) or prolonged (24 h) period followed by removal of the drug and re-suspension in drug-free medium for an additional 72 h. Cell survival was quantified by the Alamar Blue fluorometric assay for mitochondrial activity and the results reported as IC_{50}s estimated from drug dose response curves. Cells were also examined by phase microscopy at 100× before and after drug treatment to determine if exposure to the porphyrins affected cell morphology. Photosensitization by carboranylporphyrins was tested by irradiating drug treated cells for 10 min with light from a broad spectrum (600–700 nm) red light source, for a total light dose of 9.0 J/cm^2. The effect of light plus drug on cell proliferation was assayed as above using Alamar Blue.

Cellular uptake/efflux studies

Log phase cells ($2–3\times10^6$ cells per 25 cm^2 culture flask) were exposed to graded concentrations (1–100 μM) of carboranylporphyrins for 1 to 24 h to investigate concentration- and time-dependent drug accumulation. At selected time points unbound drug was removed by aspiration and three washes with HBSS. Washed cell monolayers were treated with 0.02% Triton X-100, subjected to a freeze–thaw cycle, and further dissolved in an extraction medium composed of 50% ethanol and 5 mM HEPES, pH 7. The fluorescent excitation/emission optima were determined for each porphyrin in this solvent and these wavelengths were used to measure cell-associated drug by spectrofluorometry. Retention of cell-bound porphyrin by live cells was evaluated by placing drug-treated cells into drug-free medium for various time intervals followed by extraction of residual porphyrin as above. Duplicate flasks were used to obtain an accurate cell count and results were expressed as relative carboranylporphyrin fluorescence per million cells. In selected cases, the serum component of the culture medium was varied between 1 and 10% to investigate the possible influence of serum protein and lipoproteins on carboranylporphyrin uptake and retention.

Intracellular localization of *nido*-carboranylporphyrins

Cells were grown to near confluence on 22×22 mm acid-washed glass cover slips in 35 mm culture dishes with 2 mL of standard culture medium. Porphyrins were added to final concentrations of 2–5 μM. Cells were incubated in the dark for 6–24 h. Cover slips were rinsed with HBSS to remove unbound/unincorporated porphyrin and mounted with a drop of culture medium in a latex gasket chamber on a standard 1×3 inch glass microscope slide. The cells remained viable for several hours under these conditions if kept at 37 °C and protected from direct light. Live cells were examined immediately using confocal fluorescence microscopy using 568 nm light from a krypton laser source.

Determination of intracellular boron by ICP-MS

Cells exposed to test carboranylporphyrins were washed, trypsinized, and pelleted by centrifugation. Cell pellets were re-suspended in nitric acid/hydrogen peroxide and digested in closed Teflon vessels (MLS 900, Milestone, Leutirch, Germany). A beryllium solution (10 ng/mL) was added as an internal standard.

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